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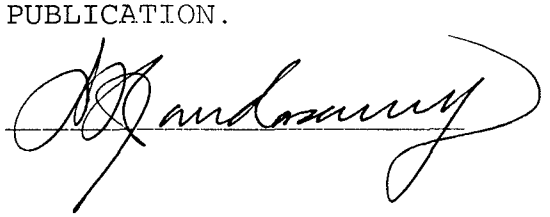

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13. ABSTRACT *(Maximum 200 Words)*
Over the past three years, I have been investigating the biochemical functions of the *Cripto* gene, which has been implicated in autocrine/paracrine signaling during human breast carcinogenesis. Since previous studies have suggested that *Cripto* encodes a growth factor-like molecule, I initially studied the binding of Cripto to mammary cell lines to examine the distribution of putative receptors. However, recent molecular genetic evidence has emerged that Cripto instead may act as a co-receptor for signaling by Nodal, a member of the transforming growth factor-beta family. To investigate this model at the biochemical level, I have expressed soluble forms of Cripto and Nodal in transfected mammalian cells, and have used co-immunoprecipitation approaches to demonstrate a direct binding interaction between Cripto and Nodal. These findings suggest that Cripto functions through modulation of Nodal signaling in mammary carcinogenesis.

14. SUBJECT TERMS
(1) growth factor receptor; (2) mammary gland development
(3) histochemical affinity reagent; (4) ligand-receptor interaction

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Table of Contents

Introduction:

Work in our laboratory on breast development and tumorigenesis has focused on the *Cripto* gene, which encodes an extracellular protein that is a member of the *EGF-CFC* gene family. Several lines of evidence have indicated that *Cripto* is involved in autocrine or paracrine signaling during human breast carcinogenesis (reviewed in (Salomon et al. 1999)). In particular, *Cripto* is overexpressed in a large percentage of human breast cancers, but not in normal breast tissue (Qi et al. 1994), and *Cripto* has transforming activity when overexpressed in NOG-8 mouse mammary epithelial cells (Ciardiello et al. 1991). To investigate the role of *Cripto* in breast development and tumorigenesis, I have been analyzing the molecular mechanisms of Cripto protein activity, with the objective of identifying a putative Cripto receptor(s).

Body:

The function of *Cripto* was initially of interest because its overexpression was found in approximately 80% of human breast carcinomas, as well as in colorectal and pancreatic cancers. Previous studies have suggested that Cripto has growth factor activity, and that addition of exogenous Cripto protein to mammary epithelial cells lines can result in tyrosine phosphorylation of the ErbB4 receptor and activation of the Ras/MAPK signaling pathway. However, since Cripto does not bind to any of the four known members of the *ErbB* receptor family, it has been unclear how Cripto might function as a ligand to activate receptor-mediated signaling.

Over the past three years, I have made significant progress towards understanding the molecular mechanisms of *Cripto* function, which has contributed to a substantial revision of our initial view of *Cripto* encoding an EGF (epidermal growth factor)-related growth factor. Based on my work and recent data from others, it is now evident that *Cripto* may in fact encode a co-receptor rather than a ligand, and is essential for signaling by a divergent member of the TGF-beta (transforming growth factor-beta) superfamily known as Nodal.

Review of recent findings in the field

Members of the *EGF-CFC* gene family include mammalian *Cripto* and *Cryptic,* frog *FRL-1,* and zebrafish *oep,* which encode proteins that share an N-terminal signal sequence, a variant EGF-like motif, a novel conserved cysteine-rich domain (the CFC (*Cripto*, *Frl-1,* and *Cryptic*) motif), and a C-terminal hydrophobic region (reviewed in (Shen and Schier 2000)). These genes encode extracellular proteins that are localized to the surface of transfected cells (Shen et al. 1997; Zhang et al. 1998). This association is mediated by the C-terminal hydrophobic domain, which in the case of Cripto is required for glycosyl-phosphatidylinositol (GPI) linkage to the cell membrane (Minchiotti et al. 2000). Notably, all *EGF-CFC* family members appear to have functionally similar activities in assays for phenotypic rescue of *oep* mutant fish embryos by mRNA microinjection (Gritsman et al. 1999).

Work from our laboratory has provided essential insights into the biological functions and potential biochemical activities of EGF-CFC proteins through analysis of knock-out mice for *Cripto* and *Cryptic* (reviewed in (Shen and Schier 2000)). We have found that *EGF-CFC* genes function in axis formation at early stages in the mouse embryo, so that *Cripto* is required for correct orientation of the A-P (anterior-posterior) axis, while *Cryptic* is necessary for determination of the L-R (left-right) axis (Ding et al. 1998; Yan et al. 1999). In contrast to previous models for Cripto having growth factor activity (Salomon et al. 1999), these and other recent lines of evidence indicate that EGF-CFC proteins act as essential co-factors for a signaling factor known as Nodal (reviewed in (Schier and Shen 2000)). The *Nodal* gene encodes a divergent member of the TGF-

beta superfamily, and displays a mutant phenotype similar to that for *Cripto* (Zhou et al. 1993; Conlon et al. 1994). The downstream signaling pathway for *Nodal* has primarily been suggested by our current understanding of TGF-beta signal transduction pathways (Massagué 1998), and by the similar and/or synergistic phenotypes for targeted disruption of *Nodal,* the type II activin receptor *ActRIIB,* the type I receptor *ActRIB,* and the cytoplasmic signal transducer *Smad2* (Schier and Shen 2000); however, there is no biochemical evidence at present that Nodal protein directly binds to and activates activin receptors.

Taken together, these findings support a model in which EGF-CFC proteins form membrane-associated components of a receptor complex, and mediate *Nodal* signaling through signal-transducing partner(s) that include activin receptors (Gritsman et al. 1999; Shen and Schier 2000). Based on the requirement of *EGF-CFC* activity for Nodal signaling, the cell-autonomy of *oep* function in zebrafish, and the localization of EGF-CFC proteins at the cell surface, EGF-CFC proteins may function as co-receptors for Nodal. Thus, EGF-CFC proteins might interact with Nodal proteins to form a complex that binds to Activin-like receptors; alternatively, EGF-CFC factors could modify or induce conformational changes in either Nodal signals and/or Activin-like receptors that allow them to interact. In principle, release of EGF-CFC proteins from the membrane might result in a freely diffusible protein, which could form part of a receptor complex on a neighboring cell that may not itself express the *EGF-CFC* gene, in effect acting as a signal. Furthermore, the downstream signaling effects documented for Cripto protein might represent cross-talk between EGF receptor and Smad2 signaling pathways (de Caestecker et al. 1998; Kretzschmar et al. 1999).

Technical Objective I: Expression patterns of *Cripto* and *Cripto* receptor

Strategy: At the start of our work on the *Cripto* gene, the existing published data suggested that Cripto was a EGF–like growth factor that functions through a novel receptor. Therefore, to analyze the functions of *Cripto,* I originally proposed to survey the distribution of endogenous *Cripto* expression as well as Cripto-AP binding sites in normal mammary tissue obtained from virgin, pregnant, lactating, and involuting mice. Furthermore, it had been shown that human Cripto protein (hCripto) can bind with high affinity to a range of mammary epithelial cell lines, including MDA-MB-453, SK-BR-3 human breast cancer cells and normal mouse HC-11 cells, and can activate p42/44 MAP kinase in these cell lines (Brandt et al. 1994; Kannan et al. 1997). These data suggested that these cell lines expressed putative Cripto receptors.

Results: To examine the expression of *Cripto* at various stages of mammary development, I employed RT-PCR and ribonuclease protection assays to analyze mammary glands from virgin, pregnant, lactating, and regressing stages. As described in my 1999 annual report, these results indicated that *Cripto* is expressed at extremely low levels throughout mammary development, with slightly elevated expression during pregnancy and lactation (data not shown). Consequently, future studies should employ a different approach to investigate the levels and distribution of *Cripto* transcripts in the mammary gland, using our gene targeted mice that contain a *LacZ* reporter gene "knocked-in" under the transcriptional control of the *Cripto* promoter (Ding et al. 1998). These mice will allow *Cripto* expression to be examined by beta-galactosidase staining of mammary glands of *Cripto-LacZ*$^{+/-}$ heterozygotes.

In order to study the interaction between Cripto protein and its binding sites on mammary epithelial cell lines as well as tissues, I generated a soluble receptor affinity reagent that consists of human Cripto protein fused to secreted human placental alkaline phosphatase (AP) (Fig. 3A). The AP-Cripto fusion protein can be effectively expressed and secreted into conditioned media of transiently transfected COS7 cells.The advantage of using a AP fusion protein as a receptor affinity

reagent is that it is non-radioactive and sensitive, and that the AP fusion protein containing conditioned media can be directly used to visualize receptor binding on cell lines by histochemical staining (Berger et al. 1988; Flanagan and Leder 1990). Since human placental AP is heat-stable, endogenous AP activity can be readily inactivated by heating.

Using these AP-Cripto conditioned media, I established quantitative assays for Cripto binding to putative receptors. In this assay, I demonstrated that AP-hCripto protein specifically binds to the human mammary cell lines MDA-MB-453 and SK-BR-3 and that the binding is saturable (Fig. 3B). Scatchard analysis using MDA-MB-453 cells reveals a K_d of 10 nM for AP-hCripto binding to its binding site and approximately $2.4x10^5$ binding sites present on each cell (Fig. 3C).

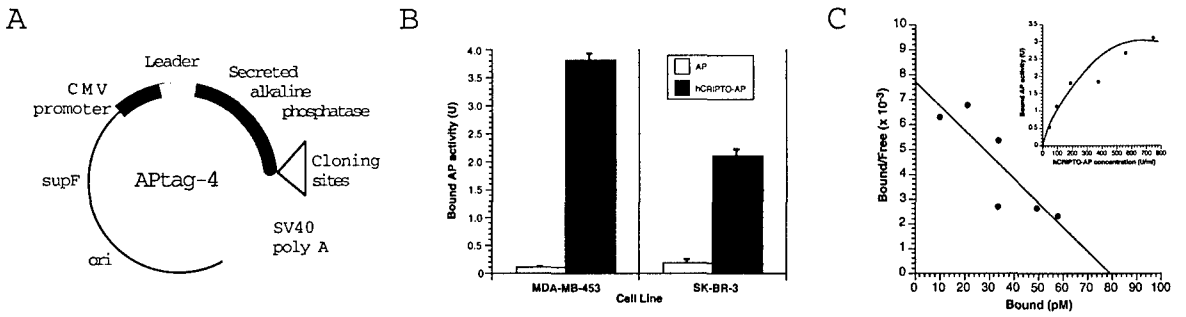

Figure 3. Binding of AP-Cripto to cultured cells. (A) Schematic map of *pAPtag-4* expression vector. The *Cripto* gene was fused in frame to the 3' end of a DNA sequence encoding the intact secretory placental alkaline phosphatase. The CMV promoter can direct high level expression of the fusion protein gene in COS7 cells. **(B) Quantitative binding of AP-Cripto to human breast cancer cell lines.** Binding assays were performed using MDA-MB-453 and SK-BR-3 cells and AP-hCripto conditioned media. AP conditioned media prepared using the *pAPtag-4* vector was used as a control. The AP activities of both AP-hCripto and AP conditioned media were adjusted to same level (750U/ml). Each assay was performed in triplcate. **(C) Scatchard analysis of AP-hCripto binding to its receptor on MDA-MB-453 cells.** Quantitative binding assays were performed using serial AP-hCripto conditioned media dilutions with different concentrations of AP activity. Each concentration was assayed in triplicate. Scatchard analysis indicated a K_d of 10 nM and 2.4 $x10^5$ receptors per cell. Calculations were based on 1 pmol AP fusion protein equal to 30 units AP activity (Cheng and Flanagan 1994). Primary data are shown in inset.

Heparin binding of Cripto. Since our laboratory focuses on mouse models, I was particularly interested in examining binding to the mouse Cripto receptor(s). For this purpose, mouse mammary HC-11 cells and AP fusions with mouse Cripto protein (AP-mCripto) were used for binding assays. Surprisingly, I found that while AP-hCripto can bind to HC-11 cells with high affinity, AP-mCripto did not (Fig. 4A). In particular, the level of AP-mCripto binding to HC-11 cells was at least 20-fold lower than that of AP-hCripto.

Because some growth factors in the EGF family have been shown to bind heparin, I investigated the possibility that human Cripto could bind to heparan sulfate, a common component of the extracellular matrix, while mouse Cripto did not. My results from a heparin bead binding assay showed that this was the case (Figure 4B). The AP-hCripto strongly binds to heparin beads, but the binding of AP-mCripto was similar to that for the AP4 control. Therefore, these data indicate that human Cripto and mouse Cripto differ in their ability to bind heparin.

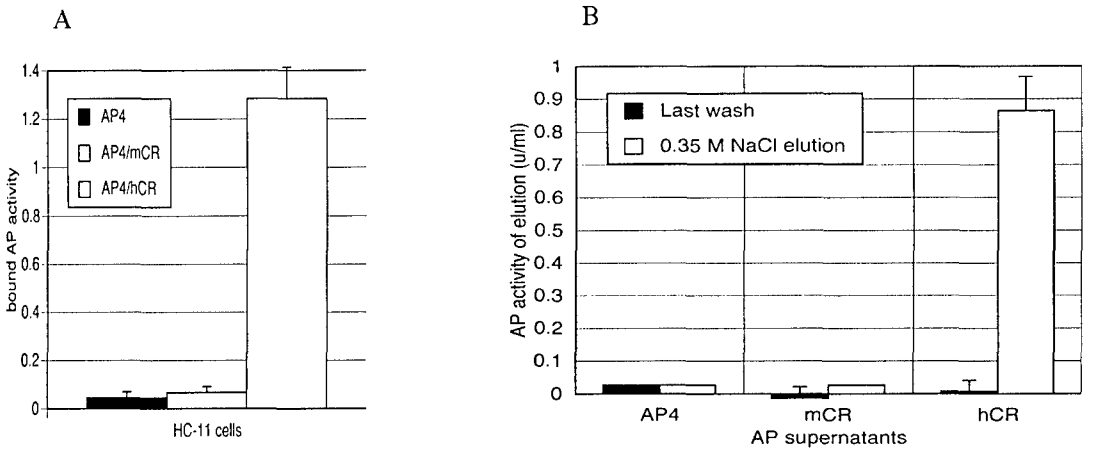

Figure 4. Quantitative binding assay. (A) Binding of mouse Cripto and human Cripto to HC-11 Cells. $3.5x10^5$ HC-11 cells were suspended in 1 ml AP-mCripto or AP-hCripto conditioned media (2000 U/ml). After incubation and wash, the cells were lysed. The cell lysates were heated at 65°C for 10 minute to inactivate endogenous AP activity and then the bound AP activity was detected as described (Flanagan and Leder 1990). AP conditioned media (2000 U/ml) was used as a control. **(B) Binding of mouse Cripto and human Cripto to heparin-beads.** 0.15 ml heparin-beads were mixed with 1 ml AP-mCripto or AP-hCripto conditioned media (1000 U/ml) and incubated at room temperature for 60 minutes. After washing with 1xPBS, the bound AP-Cripto was eluted with 0.3 ml 1xPBS/0.35M NaCl. The AP activity in the eluates was assayed. AP4 conditioned media (1000 U/ml) was used as a control.

Based on these findings, I concluded that the binding sites detected on HC-11 cells for human Cripto are likely to correspond to heparan sulfate proteoglycans, and not to signal-transducing receptors. Therefore, I did not pursue further AP-Cripto binding studies on mammary tissue, as originally proposed, but instead focused on biochemical studies of Cripto binding interactions. In summary, my studies addressed Task 1 of Technical Objective I in the original Statement of Work, but the lack of evidence for authentic receptor interactions did not support further studies in several lines of MMTV transgenic mice (Task 2 of Technical Objective I in the Statement of Work). In the meantime, the findings from our lab and others that Cripto is likely to function as a co-receptor for Nodal led me to focus on analyzing biochemical interactions with Nodal, as described below.

Technical Objective II: Cloning and characterization of the *Cripto* receptor

Strategy: In my original proposal and Statement of Work, I described a methodology to clone putative Cripto receptors using AP-fusion protein reagents to screen expression libraries from HC-11 and MD-MBA-453 cells. However, based on our heparin binding studies described above, as well as on preliminary expression library screening results that were negative (data not shown), I decided to pursue an alternative approach to identify Cripto binding proteins.

Results: In my initial experiments, I hypothesized that the failure to detect the binding of AP-mCripto to HC-11 cells might instead be due to the low sensitivity of the AP binding assay. Therefore, I decided to use AP-mCripto conditioned media and radio-labeled HC-11 cell lysate to perform a sensitive pull-down assay to identify mouse Cripto-specific binding proteins.

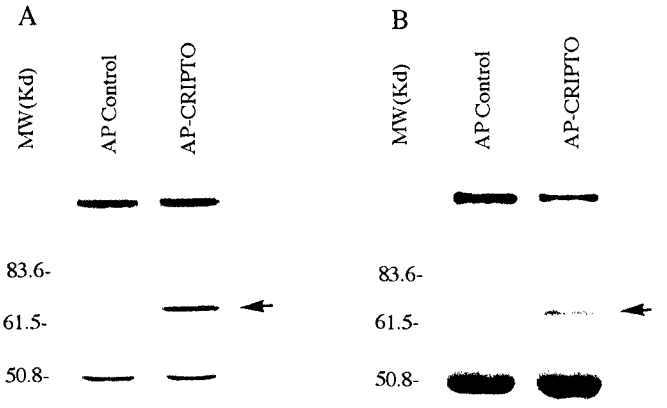

Figure 5. AP pull-down assay to identify mouse Cripto binding protein. (A) Pull-down assay using radio-labeled HC-11 cell lysate. 0.5 ml radio-labeled HC-11 lysate were mixed with 0.3 ml AP-mCripto conditioned media or AP4 conditioned media (2500 U/ml) as a control. After incubation at 4°C for 60 minutes, AP-mCripto or AP were immunoprecipitated with 5 µl AP monoclonal antibody and 30 µl Staph A cells. The precipitates were resolved by 10% SDS-PAGE. **(B) Pull-down assay using biotin-labeled HC-11 cell lysate.** HC-11 cells were labeled with 0.25 mg/ml sulfo-NHS-LC-biotin which only biotinylates cell surface proteins and then were lysed with 1xRIPA buffer. The immunoprecipitation assay was performed as for radio-labeled HC-11 cells. The immunoprecipitates were resolved by 10% SDS-PAGE, transferred onto PVDF membrane, and detected with avidin-HRP conjugate (Altin and Pagla 1995).

In this pull-down assay, ^{35}S-methionine labeled HC-11 cell lysate was mixed with AP-mCripto conditioned media or AP conditioned media as a control, and incubated at 4°C for 1 hour. AP monoclonal antibody was used to immunoprecipitate AP-mCripto or AP4 proteins, and the radio-labeled proteins co-immunoprecipitated with AP-mCripto or AP4 were identified by running a SDS-PAGE and exposing to X-ray film. Two protein bands, a major 70 Kd band and a weaker and slightly lower band, were detected for specifically binding to AP-mCripto but not AP4 (Fig. 5A). This finding suggested that these two proteins might be mouse Cripto-specific binding proteins.

To ask whether the two proteins were cell surface proteins, I used sulfo-NHS-LC-biotin, which only biotinylates cell surface proteins, to label HC-11 cells (Altin and Pagla 1995). Using the biotin-labeled HC-11 cell lysates, the same pull-down assay as described for the radio-labeled lysate was performed. The immunoprecipitates were resolved by SDS-PAGE, transferred onto PVDF membrane, and detected with avidin-HRP conjugate. In this assay, two AP-mCripto binding proteins were identified with the same molecular weight as the two detected in the radio-labeled pull-down assay (Fig. 5B), suggesting they are cell surface proteins.

Interaction of Nodal protein with Cripto and Oep proteins. During the course of this work, genetic studies in our lab as well as others showed that EGF-CFC proteins are required for Nodal signaling. In particular, Cripto and Oep proteins have been shown to be membrane-associated proteins. Based on these findings, it was suggested that EGF-CFC proteins act as essential co-factors or co-receptors for Nodal signaling, perhaps by mediating the binding of Nodal to its signal transducing receptors (type I and II activin receptors) through protein-protein interactions. Therefore, I decided to investigate the potential interaction between Nodal and EGF-CFC proteins.

For this purpose, I made constructs for expressing C-terminal truncated CRIPTO (mCRA) containing HA or Flag epitope tags; the C-terminally truncated Cripto and Oep constructs were

used to obtain efficient secretion into conditioned media. My results showed that Cripto and Oep proteins can be effectively produced by 293T cells; however, the mCRA-HA proteins expressed from the pcDNA3 vector differs from that produced using pSecTagB vector, which contains an efficient signal sequence cleavage site. Thus, pcDNA3 expressed mCRA-HA showed a single 17 Kd major band on a Western blot, while pSecTagB expressed mCRA-HA had four bands ranging from 15-20 Kd in size (Fig. 6A), perhaps because the pSecTagB vector uses the heterologous Ig κ chain signal sequence, which may result in different protein processing. I chose to use the pSecTagB vector for protein expression because it resulted in a significantly higher expression level than pcDNA3 (Fig. 6A). The Flag-Oep m134 protein produced by 293T cells migrated as a doublet with a 14-16 Kd sizes (Fig.6B).

In addition, I also showed that Nodal-Flag can be processed and secreted by 293T cells. The conditioned media produced by 293T cells expressing Nodal-Flag contained unprocessed 38 Kd Nodal as well as processed, mature 14 Kd Nodal proteins (Fig. 6C). In contrast, when I used COS7 cells to express Nodal protein, only unprocessed Nodal protein can be detected in cell lysates and not in the conditioned media, suggesting that Nodal protein processing and secreting is cell-line dependent (Fig. 6D). Furthermore, I also found that the Nodal-Flag protein was largely aggregated in the conditioned media of 293T cells, since most of the Nodal-Flag protein migrated at apparent molecular weights of over 200 Kd on non-reducing SDS-PAGE gels. However, other data from sucrose gradient analysis as well as biological activity assays in the chick suggested that there was Nodal activity present in these conditioned media, so I used these for further analysis in co-immunoprecipitation experiments.

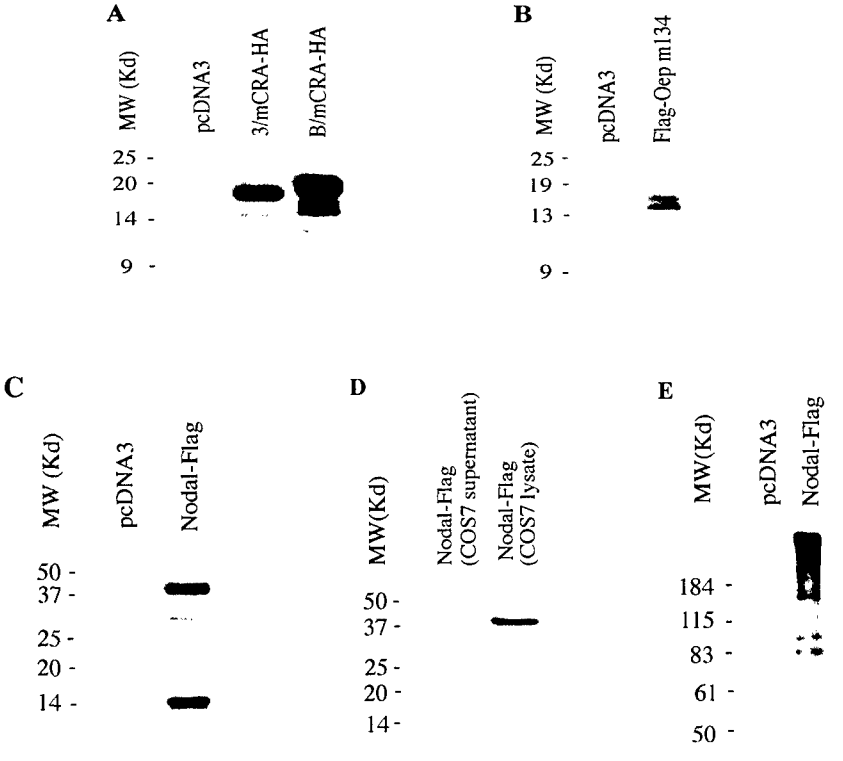

Figure 6. Expression of Nodal, Cripto, and Oep proteins by 293T or COS7 cells. Transiently transfected 293T or COS7 cells were cultured in serum free Optimem medium, and protein expression levels in conditioned media were examined by Western blotting. *pcDNA3* transfected culture supernatant was used

as a control. **(A) Expression of mCRA-HA protein.** *pcDNA3/mCRA-HA* and *pSecTagB/mCRA-HA* were transfected into 293T cells and conditioned media collected. mCRA-HA protein were detected by Western blotting (10 µl conditioned media/lane) with anti-HA monoclonal antibody (Invitrogen). **(B) Expression of Flag-Oep protein.** *pFlag-Oep^{m134}* was used to express C-terminally-truncated Oep in 293T conditioned media, followed by detection by Western blotting (10 µl conditioned media/lane) using anti-Flag monoclonal antibody (Sigma). **(C) Expression of Nodal-Flag in 293T cells.** *pcDNA3/Nodal-Flag* was used to express Nodal in 293T conditioned media; Nodal expression was detected by Western blotting (16 µl/lane) using anti-Flag monoclonal antibody. **(D) Expression of Nodal-Flag by COS7 cells.** was transfected into COS7 cells, followed by Western blotting of conditioned media and cell lysates with anti-Flag monoclonal antibody (15 µl conditioned media or cell lysate/lane). **(E) Aggregation of Nodal-Flag.** Conditioned media (8 µl) from 293T cells transfected with *pcDNA3/Nodal-Flag* was analyzed on a non-reducing SDS-PAGE gel, followed by Western blotting with anti-Flag monoclonal antibody.

Consequently, I used conditioned media containing epitope-tagged Nodal, Cripto, and/or Oep proteins to perform co-immunoprecipitation assays to detect interaction of Nodal with Cripto or Oep. To detect interaction between Nodal and Cripto, mCRA-HA conditioned media expressed following transfected by *pcDNA3/mCRA-HA* or *pSecTagB/mCRA-HA* was mixed and incubated with Nodal-Flag conditioned media. mCRA-HA protein in the mixture was immunoprecipitated by adding anti-HA-beads, and immunoprecipitated complexes were resolved on a 13.5% SDS-PAGE gel and detected by Western blotting using anti-Flag monoclonal antibody. My results showed that Nodal-Flag can be co-immunoprecipitated with mCRA-HA (Fig. 7A), and similarly, Nodal-HA can be co-immunoprecipitated with Flag-Oep (Fig. 7B).

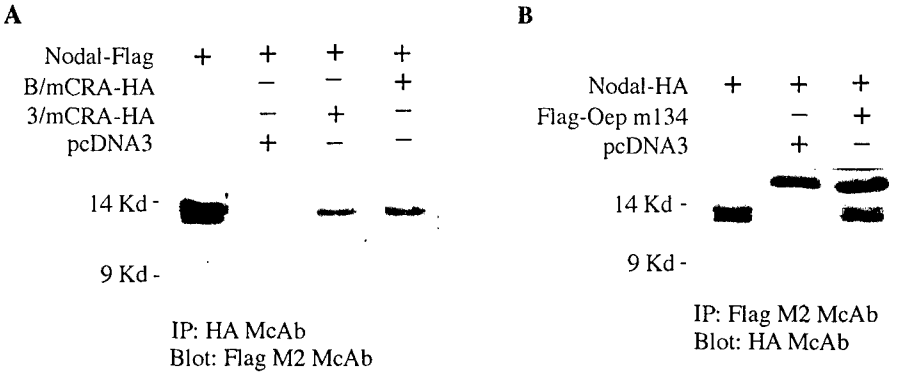

Figure 7. Co-immunoprecipitation assays for detecting interaction between Nodal and EGF-CFC proteins. (A) Interaction between Nodal and Cripto proteins. 0.32 ml mCRA-HA conditioned media was mixed with 0.64 ml Nodal-Flag conditioned media. After incubation, 20 µl anti-HA monoclonal antibody (McAb) coupled beads were added to immunoprecipitate mCRA-HA protein. The immunoprecipitated complexes were resolved on a 13.5% SDS-PAGE gel, and Western blotting was performed using anti-Flag monoclonal antibody. (B) Interaction between Nodal and Oep proteins. 0.5 ml Flag-Oep^{m134} and Nodal-HA conditioned media were co-immunoprecipitated using 60 µl anti-Flag McAb-beads, followed by Western blotting using anti-HA monoclonal antibody.

In summary, based on my findings, I have provided biochemical evidence for a direct interaction between Nodal and EGF-CFC proteins. These experiments support the current model that Cripto represents a co-receptor for the Nodal ligand, and are not consistent with previous views of Cripto as a growth factor-like molecule. Thus, while I did not pursue the specific experiments in Tasks 1 and 2 of Technical Objective II in the original Statement of Work, I successfully

accomplished the identification of a Cripto-interacting protein (Task 3 of Technical Objective II in the Statement of Work), and thereby addressed the overall goal of this specific aim.

Key Research Accomplishments:

- I have established an alkaline-phosphatase fusion protein system for assaying the interaction of Cripto with cell-surface proteins.
- I have demonstrated that mouse Cripto is a heparin-binding protein.
- I have expressed soluble Cripto and Nodal proteins in conditioned media from transfected mammalian cell lines for analyses of their activities and potential biochemical interactions.
- Co-immunoprecipitation analyses of these proteins has provided evidence of a direct binding interaction.
- My findings raise the possibility that *Cripto* overexpression in human breast cancer may result in de-regulation of TGF-beta signaling pathways.

Reportable Outcomes:

Published manuscripts:

None.

Meeting abstracts:

Shen, M. M., E, C., Saplakoglu, U., Yan, Y.-T., and Ding, J. (2000). Functional analysis of *EGF-CFC* genes in mouse development indicates their essential role in Nodal signaling. Era of Hope Department of Defense Breast Cancer Research Program Meeting. (Atlanta, GA).

E, C., Saplakoglu, U., and Shen, M. M. (2000). Role for Cripto as a cofactor in the Nodal signaling pathway. Era of Hope Department of Defense Breast Cancer Research Program Meeting. (Atlanta, GA).

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Chaosu E

Minjung Kim

Degrees obtained supported by this award:

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Conclusions:

Although *Cripto* was the first member of the *EGF-CFC* gene family to be identified, its biochemical function has been poorly understood. Thus, it has been significant that recent genetic data from mouse and zebrafish studies has indicated that EGF-CFC proteins function as co-factors

or co-receptors for the TGF-beta factor Nodal. In my work, I have expressed soluble Nodal and Cripto proteins in conditioned media from transfected mammalian cells to detect a physical interaction between Nodal and EGF-CFC proteins. My data support a direct association between Nodal and Cripto in vivo to facilitate Nodal signaling, presumably through activin receptors. At present, these data do not allow us to distinguish whether Cripto acts as a co-factor or as a co-receptor for Nodal, however our ongoing studies should be able to resolve this critical issue. Finally, this work suggests that the activity of soluble Cripto on mammary epithelial cell lines is due to activation of a Nodal signaling pathway, and indicate the necessity of further studies on Nodal signaling in breast carcinogenesis.

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DEPARTMENT OF THE ARMY
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 26 Aug 02

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

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